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[54] **PROCESS FOR THE EXTRACTION OF
POLYUNSATURATED FATTY ACID ESTERS
FROM FISH OILS**

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[63] Continuation-in-part of Ser. No. 199,306, May 26,
1988, abandoned.

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554/175; 554/193; 554/206; 554/210; 554/224

[58] **Field of Search** 424/523; 260/412, 413,
260/419, 420, 421, 423, 424, 425, 426, 427, 428,
428.5

[56] References Cited**U.S. PATENT DOCUMENTS**

4,164,506	8/1979	Kawahara et al.	260/421
4,179,454	12/1979	Mehta et al.	260/419
4,377,526	3/1983	Fujita et al.	260/424
4,438,106	3/1984	Wagu et al.	514/58
4,526,902	7/1985	Rubin	514/560
4,554,107	11/1985	Takao	260/421
4,675,132	6/1987	Stout et al.	260/410.9 R
4,792,418	12/1988	Rubin et al.	260/420

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[57] ABSTRACT

A process for the extraction of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid esters from crude fish oils, by means of transesterification with ethanol and H₂SO₄ and two-step molecular distillation.

2 Claims, No Drawings

PROCESS FOR THE EXTRACTION OF POLYUNSATURATED FATTY ACID ESTERS FROM FISH OILS

This application is a continuation-in-part of application Ser. No. 199,306 filed May 26, 1988 now abandoned.

The present invention relates to a process for the preparation of a mixture of fatty acid alkyl esters having a high concentration in eicosapentaenoic and docosahexaenoic acids, starting from fish oils of various origin, and to pharmaceutical and/or dietetic compositions containing said mixture.

Moreover, the process according to the invention is suited to deodorize and deacidify said oils, in view of any dietetic and/or alimentary use.

Polyunsaturated fatty acids are known to play two important roles in human physiology: a structural role, as constituents of cell membrane phospholipids, and a functional role, as Prostaglandin precursors.

In fact, fatty acids of the α -linolenic acid family have a basic role in development and function of brain, retina and gonads, as well as the formation of PGI_3 and TxA_3 , which are factors of paramount importance for the anti-platelet aggregating activity.

Among these, particularly important are the long chain members of the ω -3-family, i.e. eicosapentaenoic (20:5 ω -3) or EPA and docosahexaenoic (22:6 ω -3) or DHA acids, deriving from desaturation and elongation of α -linolenic acid, thanks to the intervention of the related enzymes (Δ -desaturase).

EPA, as the precursor of PGI_3 and TxA_3 , exerts an anti-platelet aggregating activity and an anti-thrombotic effect which can be related to cyclooxygenase inhibition (aspirine-like effect) and/or to the competition with arachidonic acid for said enzyme, with an accordingly decreased synthesis of PGI_2 and TxA_2 , which are known platelet aggregating agents.

DHA is the most important component of human lipids and brain and is present in high concentrations in synaptic membranes phospholipids, which may imply a role in nervous impulse transmission. Moreover, DHA being a structural element of platelet cell, it indirectly exerts an important role in anti-thrombotic action, due to the increase in platelet fluidity.

Recent studies evidenced a decrease in Δ -6 desaturase in man as the age goes on (after 35 years); said phenomenon causes thus an endogenous lack in the above mentioned acids, which therefore should be administered through diet or by means of suitable compositions. However, various practical difficulties opposed up to now a wide use of said acids in therapy or as alimentary integrators, which use on the other hand should be highly desirable, in view of the above reported biochemical and pharmacological considerations.

Said difficulties mainly relate to extraction of said acids from fish oils, purification and concentration to values convenient for the pharmaceutical use and deodorization thereof.

Even though a number of methods have already been proposed and disclosed, the above objects have still not been attained satisfactorily, as proved, inter alia, by the still restricted use of EPA and/or DHA, in spite of the remarkable potentialities thereof as drugs or alimentary integrators. The methods up to now known, which are based on different techniques such as degreasing, countercurrent extraction, urea addition, liquid chromatog-

raphy, and distillation, give rather low yields and products which easily deteriorate if exposed to air or light. Moreover, the major part of the known methods refers to purification of only eicosapentaenoic acid, to the detriment of other useful unsaturated acids, such as DHA.

As an example, U.S. Pat. No. 4,377,526 discloses a process for purification of EPA or the esters thereof, which comprises treatment with urea, followed by fractional distillation. By said method, EPA percentages higher than 70% are obtained, whereas DHA is present only as a residue (3-5%).

More recently, U.S. Pat. Nos. 4,554,107 and 4,623,488 disclosed a purification method based on the technique known as molecular distillation: in this case, a deodorized fish oil is obtained which is enriched in EPA and DHA, in rather low yields (30%), due to the drastic conditions used.

A first object of the invention is therefore provided by a method for the extraction of DHA and EPA ethyl esters from crude fish oils in high yields, under conditions which can easily be applied on industrial scale, which give a stable and odourless product, which can be used in human therapy or as a dietetic and alimentary integrator.

A second object of the invention, in fact, is provided by pharmaceutical or alimentary compositions containing as the active ingredient a mixture of EPA and DHA ethyl esters, for the treatment or the prophylaxis of cardio-vascular diseases.

According to one embodiment of the present invention, it has been found that highly purified mixtures having a total EPA/DHA ethyl ester content of at least about 65% can be obtained starting from crude fish oil by subjecting the latter to transesterification with ethanol in the presence of a catalytic amount of sulfuric acid, extracting the reaction product with a hydrocarbon, e.g., cyclohexane, and then subjecting the extract to silica gel chromatography and multi-step, i.e., at least two-step, molecular distillation under controlled conditions.

According to a second embodiment of the invention, a mixture in which the DHA ethyl ester content is 85-95%, and even higher, can be obtained by dissolving the reaction product, obtained from the silica gel chromatography, in acetone accompanied by slow cooling and then subjecting the residue obtained by filtration and removal of the solvent to multi-stage molecular distillation.

The process of the invention can be easily carried out on industrial scale, and is characterized in that it consists in a surprisingly low number of steps, if compared with the processes up to now known, which use crude fish oils as the starting material. Moreover, it should be stressed as particularly surprising that enriching in EPA and DHA and deodorizing are simultaneously attained by a molecular distillation technique, which has been hitherto considered merely for the purpose of deodorization, with completely different operative parameters.

In fact, the above cited U.S. patents carry out three-steps molecular distillation on long chain unsaturated acid triglycerides, using drastic temperature conditions (up to 260°-300° C.), operating in the presence of two additives (glycerol and momooleylglyceride) in order to fluidize the liquid to be distilled.

On the contrary, the process object of the present invention is characterized in that it is carried out on the ethyl ester mixture instead of the triglycerides; more-

over operative conditions are much milder (only two steps at a lower temperature) and give higher yields, mainly in DHA which is known to be less stable.

According to the invention, deodorization is effected in the first step and the products responsible for the bad smell are removed by the low temperature trap, upstream the pump. This allows to operate on crude oil not previously depurated, which, besides being effectively deodorized, is also deacidified, to make it suited for alimentary use.

A preferred EPA and DHA source consists in oils deriving from working of "blue fish", such as anchovies, sardines, cods, mackerels, herrings and the like.

The oil is diluted with ethanol, then refluxed in the presence of catalytic amounts of concentrated sulfuric acid. After extraction with hexane, the transesterification mixture is subjected to silica gel chromatography, then to a two-step molecular distillation process, with a vacuum of about 10^{-3} mm Hg and at an evaporation temperature ranging from 65° – 70° C. to 105° – 125° C. and condenser at 5° C.

The product obtained as a distillation residue has and EPA+DHA content higher than 65% and the DHA:EPA ratio, which generally depends on the starting oil, is about 3:2.

According to an alternative process of the invention, the ethanolic solution obtained by transesterification can be treated with urea, in order to remove the salts of fatty acids of lower unsaturation. However, said further step generally is not necessary, since in the major part of cases the only molecular distillation alone is sufficient to attain the desired effects.

According to a further aspect of the process of the invention, it is also possible to obtain docosahexaenoic acid having an assay as high as 85–95%, according to the starting fish oil used. For this purpose, the ester mixture from the silica gel chromatography is dissolved in acetone and the solution slowly cooled to -40° C. The formed precipitate is then filtered, the solvent is removed under reduced pressure and the residue is subjected to molecular distillation, which is carried out in two steps, always at 10^{-3} torr, but at higher temperatures, namely 80° / 100° C. for the first step and 105° / 125° C. for the second step. A product having a DHA ethyl ester content greater than 95% can be obtained by subjecting a mixture having a 80–90%, or greater total content of EPA/DHA ethyl esters to further molecular distillation at a vacuum of 10^{-3} mm Hg and a temperature in the range of 50 – 110° C.

The product obtained by the process of the invention proved to be particularly convenient for pharmaceutical use, in form of appropriate pharmaceutical compositions. In fact, a favourable synergism was evidenced between EPA and DHA, such as to give a therapeutical effectiveness higher than that of the single components. The pharmaceutical compositions of the invention will be prepared by means of techniques and excipients conventionally used for active ingredients in form of oils, as described in "Remington's Pharmaceutical Sciences handbook", Hack Pub. Co., N.Y. USA. Preferred administration routes are the oral and the parenteral ones, whereas posology will generally range from 500 to 5,000 mg of EPA and DHA ethyl ester mixture obtained by the inventive process, depending on pathology and conditions of the patient to be treated. Anyway, higher dosages are not contraindicated, since the active ingredient is almost non-toxic. The same mixture

can be used as dietetic or alimentary integrator, optionally diluted with other appropriate vegetal oils.

In fact, the mixture obtained by the process of the invention is particularly convenient for the prophylaxis of diseases related to platelet hyperaggregation conditions, since it is completely free from linolenic acid derivatives which are precursors of arachidonic acid and accordingly of PGE_2 and TxA_2 which are factors able to oppose and make void the favourable pharmacologic properties deriving from the production of PGI_3 and TxA_3 , induced by EPA, DHA and derivatives thereof.

The following example further illustrates the invention without limiting it.

EXAMPLE 1

a) 80 kg of fish oil was dissolved in 50 l of ethanol containing 5% conc. sulfuric acid. The whole was refluxed under nitrogen for 8 hours, then cooled; ethanol excess was removed and the volume was doubled with water. At this moment an extraction was carried out with appropriate amounts of hexane. The hexane solution, after repeated washings with water, was chromatographed on a silica gel 100 column to remove impurities. The solution from this column was freed from n-hexane under vacuum, to obtain a yield of about 65 kg of esterified products. Control can be effected by G.C. (gas chromatography).

b) The product from step a) was subjected to double-step molecular distillation, under a vacuum of about 10^{-3} mm Hg, at an evaporation temperature of 65° – 70° C. and condenser temperature of 5° C.

Percentages of the fatty acids present in the oil before and after the treatment, determined by G.C., are reported hereinbelow.

Fatty acids	% Ethyl esters on crude product	% Ethyl esters on final product
$\text{C}_{14}:0$	8.4	0.1
$\text{C}_{16}:0$	16.0	1.7
$\text{C}_{16}:1\omega7$	9.8	0.3
$\text{C}_{18}:1\omega9$	9.9	3.0
$\text{C}_{18}:1\omega7$	3.1	1.0
$\text{C}_{20}:5\omega3$	9.6	29.8
$\text{C}_{22}:5\omega3$	1.0	4.1
$\text{C}_{22}:6\omega3$	10.7	39.9

EXAMPLE 2

80 kg of fish oil together with 50 kg of ethanol were placed in a closed reactor containing 2.5 kg of conc. sulfuric acid and refluxed under nitrogen for six hours at a temperature of $82^{\circ}\pm^{\circ}$ C. Control was effected by T.L.C. on silica gel plates using a mixture of petroleum ether/diethyl ether/acetic acid (85/14/1) as the eluant. The developer comprised a 1:1 mixture of conc. sulfuric acid and methyl alcohol. When the chromatographic control showed the end of the reaction, heating was discontinued and excess ethanol removed by distillation. The residue was cooled to room temperature and added to 200 kg of water 150 kg of cyclohexane. The mixture was stirred and the water discharged. Water washings were repeated until the discharge showed a neutral reaction. The cyclohexane solution was dried with anhydrous sodium sulfate and the cyclohexane removed by vacuum distillation (20 mm Hg) at 60° C. The product residue comprising EPA and DHA ethyl

esters was stored under nitrogen for subsequent molecular distillation.

EXAMPLE 3

35-40% Total Conc. Combined EPA/DHA Ethyl Esters

The product of Example 2 was subjected to molecular distillation under a pressure of 10^{-3} mm Hg and an evaporator temperature of 90° - 110° C. to remove natural and process impurities. The purified product obtained had the EPA (15-20%) concentrations originally present in the starting fish oil of Example 2.

EXAMPLE 4

40-50% Total Conc. EPA/DHA Ethyl Esters

A sample of the product of Example 3 was subjected to molecular distillation under the same pressure as in Example 3 but at a temperature of 50° C. The elimination of C_{16} and C_{18} acid ethyl esters in the distillate resulted in a product having a 40-50% total concentration of EPA/DHA ethyl esters.

EXAMPLE 5

50-60% Total Conc. EPA/DHA Ethyl Esters

The procedure of Example 4 was repeated on another sample of the product of Example 3 except that the molecular distillation temperature was 60° - 70° C. Further removal of C_{16} and C_{18} acid ethyl esters resulted in a 50-60% total concentration of EPA/DHA ethyl esters.

EXAMPLE 6

60-70% Total Conc. EPA/DHA Ethyl Esters

The procedure of Example 5 was repeated except that the temperature was 70° - 80° C. which resulted in a product having a 60-70% total concentration of EPA/DHA ethyl esters.

EXAMPLE 7

70-80% Total Conc. EPA/DHA Ethyl Esters

A sample of the product of Example 6 was subjected to molecular distillation at a pressure of 10^{-3} mm Hg and a temperature of 80° - 90° C. A product having a 70-80% total concentration of EPA/DHA ethyl esters was obtained.

EXAMPLE 8

80-90% Total Conc. EPA/DHA Ethyl Esters

To a solution of 20 kg of urea in 120 liters of ethanol were added 15 liters of the product of Example 7 and the mixture shaken while heating under nitrogen. After

cooling, the resultant precipitate was separated and the remaining solution vacuum concentrated to a small volume. After washing with water to remove all trace of urea, drying the organic solution and removing the solvent by vacuum distillation, the product was subjected to molecular distillation as in Example 3 but at an evaporator temperature of 70° - 90° C. A product of 80-90% total concentration of EPA/DHA ethyl esters was obtained by virtue of $C_{16,18,20}$ acid ethyl esters being removed in the distillate.

EXAMPLE 9

90% Conc. DHA Ethyl Ester

A sample of the product of Example 8 is subjected to double molecular distillation under conditions as in Example 3 but at an evaporator temperature of 75° - 95° C. The residue was 90% DHA ethyl ester while the distillate comprised EPA ethyl ester and minor amounts of other lower acid ethyl esters.

EXAMPLE 10

96% Conc. DHA Ethyl Ester

A sample of the product of Example 9 was subjected to molecular distillation under the same conditions as used in Example 9. The residue was 96% DHA ethyl ester while the distillate was essentially EPA ethyl ester together with minor amounts of other lower acid ethyl esters.

We claim:

1. A process for extracting an odor-free mixture of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) ethyl esters from crude fish oil said odor-free mixture having a total EPA-DHA ethyl ester content of at least 65% and a DPA ethyl ester/EPA ethyl ester ratio of at least 3:2, which consists essentially of subjecting said crude fish oil to transesterification by diluting the latter with ethanol and refluxing in the presence of a catalytic amount of sulfuric acid, extracting the transesterification reaction product and subjecting said product to silica gel chromatography followed by molecular distillation at a pressure of about 10^{-3} mm Hg and at a temperature of about 65° - 70° C.

2. A process according to claim 1 for producing a mixture having a DHA ethyl ester content of 85-95% which consists in dissolving the silica gel chromatographed reaction product in acetone with cooling to about -40° C., separating the resultant residue and subjecting it to two step molecular distribution in which the two step temperatures are 80° - 100° C. and 105° - 125° C., respectively.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:)
: Examiner P.K. Sripada
HARALD BREIVIK, et al.)
: Group Art Unit: 1202
Serial No.: 07/902,500)
: Attorney Docket No.
Filed: June 23, 1992) 1526.100 Cont. I
:
For: FATTY ACID COMPOSITION)

The Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

RULE 132 DECLARATION OF HARALD BREIVIK

I, Harald Breivik, a resident of Skjelsvik, Norway,
do hereby declare as follows:

1) Qualifications: I am one of the applicants in
the above-identified patent application (hereinafter "this
application"). I hold a Ph. D. degree in Organic Chemistry,
obtained in 1980, from the University of Oslo. From 1980
until the present I have been employed by Norsk Hydro a.s,
assignee of this application, as a Senior Scientist in its
Research Center in Porsgrunn, Norway. I currently head the
Omega-3 Fatty Acids Group at the Research Center. Attached
Exhibit A is a copy of my curriculum vitae, which accurately
reports my scientific education, training, and experience.

2) Procedure and Results: In response to the
rejection of this application, on grounds of unpatentability
over the disclosure in U.S. Patent No. 5,130,611 to Cornieri
et al., I have attempted to reproduce those specific Examples

in Cornieri et al. that disclose the preparation of concentrated mixtures of ethyl esters of C₂₀₊ fatty acids that contain a combined concentration of 80% or more of EPA+DHA. To do this, I and my colleagues followed as faithfully as possible the sequence of procedures described in Examples 2, 3, and 6-8 of the patent. Our objectives were (1) to ascertain whether those examples are operable to produce such a highly concentrated mixture of EPA+DHA and (2) if they are so operable, to ascertain what the EPA:DHA ratio is likely to be in the 80%+ resultant mixture, since that ratio is not disclosed in the examples themselves. The details of our work, and the results, are as follows:

Example 2 Replication

For practical reasons, we worked with a batch size that was half that reported in Example 2 of the patent, starting with 40 kg of fish oil, rather than 80. To a mixture of the fish oil and 25 kg of ethanol, in a closed reactor, was added 1.25 kg of concentrated sulfuric acid. The mixture was refluxed under nitrogen for six hours at a temperature of approximately 82° C. At the end of the reaction, heating was discontinued and excess ethanol was removed by distillation. The residue was cooled to room temperature and added to 100 kg of water and 75 kg of cyclohexane. The mixture was stirred and the water discharged. Water washings were repeated until the discharge showed a neutral reaction. The cyclohexane was removed by vacuum distillation at a temperature that varied from 61 to 70° C. (It was unnecessary

to also dry with anhydrous sodium sulfate, as the distillation is azeotropic and the water comes over with the cyclohexane. The H₂O content after the distillation was only 76 ppm.) The product residue comprising EPA and DHA ethyl esters, which had the following composition, was stored under nitrogen for subsequent molecular distillation:

<u>Fatty acid</u>	<u>GLC area% ^{1/}</u>
C14:0	7.3
C16:0	18.2
C16:1 n-7	8.3
Phytanate	1.5
C16:3 n-4	1.1
C16:4 n-3	1.4
C18:0	4.1
C18:1 n-7	3.5
C18:1 n-9	9.9
C18:2 n-6	1.3
C18:3 n-6	0.5
C18:3 n-3	0.7
C18:4 n-3	1.9
C20:4 n-6	1.1
C20:4 n-3	1.1
C20:5 n-3	14.6 EPA
C22:5 n-3	2.6
C22:6 n-3	10.5 DHA

Example 3 Replication

The product of Example 2 was subjected to molecular distillation under a starting pressure of 10⁻³ mbar (which is substantially the same as -- i.e., not significantly different from -- 10⁻³ mm Hg^{2/}) and an evaporator temperature

^{1/} This is an approximation of weight percent, obtained relatively quickly by gas/liquid chromatographic analysis. Later in our work, as reported herein, actual weight percentages were determined for the most relevant products.

^{2/} 1 x 10⁻³ mbar = 0.75 x 10⁻³ mm Hg. This is as low as the pressure indicators go on Leybold KDL4 stills.

of about 108° C. (within the Cornieri et al. patent's range of "90-110° C") to remove natural and process impurities. (As would be expected, the pressure increased to 10⁻² mbar once the distillation was underway.) To guard against any aberrant results that might be caused by unseen equipment malfunction or idiosyncrasies, the product was divided into four parts, each of which was separately distilled, and two different molecular distillation stills were employed. Both were Leybold Model No. KDL4 stills: one located in Porsgrunn, Norway, at the Norsk Hydro a.s Research Center, the other in Sandefjord, Norway, at the facilities of Pronova Biocare a.s, a partially owned subsidiary of Norsk Hydro. The products obtained had the following compositions:

Residue Composition--First Distillation (90-110°C)

<u>Batch</u>	<u>EPA (GLC area%)</u>	<u>DHA (GLC area%)</u>	<u>EPA+DHA</u>	<u>EPA:DHA</u>
851-4* (Table 1)	20	17.4	37.5	1.15:1
851-27 * (Tables 2 and 3)	19.9	17.1	37	1:16:1
Res. 1** (Table 4)	20	17.2	37.2	1:16:1
Res:1** (Table 5)	18.0	15.7	33.7	1.15:1

* Distilled at Porsgrunn.
 ** Distilled at Sandefjord.

Example 6 Replication

(As reported in the patent, Cornieri's Examples 4 and 5 led to concentrations of only 40-60% EPA+DHA, so we did not attempt to repeat them. Again, our objective was to achieve the 80%+ product reported in Example 8 of Cornieri et al. The patentees report that that was derived from the product of Example 7, which was in turn derived from the product of Example 6, which was prepared using the procedure of Example 5, but employing a higher evaporator temperature. Accordingly, we proceeded from Cornieri's Example 3 to his Example 6, as follows.)

Four samples of the products of Example 3 were subjected to molecular distillation, under the same starting pressure as in Example 3, but at temperatures in the range of 70°-80° C. The products had the following compositions:

Residue Composition--Second Distillation (70-80°C)

<u>Batch</u>	<u>EPA (GLC area%)</u>	<u>DHA (GLC area%)</u>	<u>EPA+DHA</u>	<u>EPA:DHA</u>
851-29* (Table 2)	26	26.1	52.1	1:1
Res:2.1** (Table 5)	21.2	18.8	40.0	1.13:1
Res:2.2** (Table 5)	23.1	27.6	50.7	0.84:1
Res:2.3** Table 5)	22.9	26.7	49.6	0.86:1

* Distilled at Porsgrunn.

** Distilled at Sandefjord.

As seen from the above results, Example 6 as replicated did not yield the 60-70% combined EPA+DHA ethyl ester concentration reported in the patent. It yielded only about a 40-52 % concentration, and that was as measured by GLC area, which tends for fish oil fatty acid mixtures to read somewhat higher than actual (or weight) percentages, especially if the composition has been repeatedly subjected to molecular distillation. This is because at distillation temperatures the polyunsaturated molecules tend to react together, forming oligomers. The high molecular weight oligomers tend not to pass through the chromatographic column, and thus are not included in the machine's calculation of the total sample size. The EPA and DHA, which do pass through the column, appear to the machine, therefore, to constitute a higher percentage of the sample than is actually the case. Nevertheless, the GLC area % analysis is useful because it can be performed quickly.

We repeated the distillation many times, altering pressures, temperatures, and feed rates, in an effort to maximize the EPA+DHA concentration. The highest that could be achieved by molecular distillation alone was 48.7%, on a weight basis. (See Batch No. 851-31 in Table 2, attached.) After several distillations of any one mixture, the EPA+DHA concentration was seen to decline, rather than increase. This is as expected, since the EPA and DHA molecules, being polyunsaturated, tend, as mentioned above, to combine to produce oligomers, the longer they are subjected to elevated

temperatures. Also, the EPA:DHA ratio became smaller and smaller, which also is as expected, since EPA is more volatile than DHA and, therefore, is preferentially removed in the distillate.

The results of all of the various distillations are reported in Tables 1-5, attached hereto.

Notes to Tables 1-5

The "A%" figures are area percentages as measured by gas/liquid chromatography. The "W%" is weight percent as determined by the analytical procedure described in Tande et al., *Journal of the American Oil Chemists Society*, 69: 1124 (1992). Because of the time involved, only selected products (as reported) were subjected to the weight percent analysis. After repeated distillations, the A% readings become less representative of the weight percent of EPA and DHA in the mixture. This is because a substantial amount of oligomers formed from the repeated heating of the mixture is retained on the GLC column, as explained above, and the A% readings are based solely on what passes through the column.

"Res." is the residue fraction, or "bottoms", from the distillation. "Dist." is the distillate fraction.

In addition to EPA and DHA, the products were also analyzed for DPA (C22:5n-3), because the concentration of the latter is known to build up significantly, in parallel with the DHA, as such mixtures are subjected to repeated distillation steps to remove the more volatile C₁₆₋₁₈ fatty acids.

In each step, the reported EPA, DHA, and DPA contents were in the residue fractions. Step 2 involved the molecular distillation of the residue from Step 1. Step 3 involved the distillation of the residue from Step 2, and so forth.

Example 8 Replication

(Cornieri's Example 7 calls for the redistillation of the product of Example 6, but at a higher temperature. That essentially is what was attempted with the repeated distillations reported above. Having reached the maximum EPA+DHA concentration achievable through molecular distillation, we then subjected that product to Cornieri's final step in the concentration process, Example 8, as follows.)

The three highest-concentration products from Example 6 were selected for further treatment according to Example 8. Each product was added to a solution of urea in ethanol, and the mixture was shaken while heating under nitrogen. The ratio of ingredients was 1 g. of urea, to 6 ml. of ethanol, to 0.75 ml. of product (the same as 20 kg of urea in 120 liters of ethanol with 15 liters of product). After cooling, the resultant precipitate was separated and the remaining solution was vacuum concentrated to a small volume. After washing with water to remove all traces of urea, drying the organic solution, and removing the solvent by vacuum distillation, the product was subjected to molecular distillation as in Example 3, but at an evaporator

temperature in the range of 70-90° C. The composition of the resultant products, before and after the final molecular distillations, were as follows (also described in Tables 2, 3, and 4). These are true weight amounts, not the GLC area percentages:

Composition -- After Urea Fractionation and Final Molecular Distillation					
<u>Batch</u>	<u>Stage</u>	<u>EPA (Wt.%)</u>	<u>DHA (Wt.%)</u>	<u>EPA+DHA</u>	<u>EPA:DHA</u>
851-39 I (Table 2)	Pre-Distillation	10.8	43	53.8	0.25:1
851-39 II* (Table 2)	Post-Distillation	9.7	43	52.7	0.23:1
851-46 I (Table 3)	Pre-Distillation	13.7	41.4	55.1	0.33:1
851-46 II* (Table 3)	Post-Distillation	12.2	41.9	54.1	0.29:1
851-41 I (Table 4)	Pre-Distillation	12.2	40.9	53.1	0.3:1
851-41 II* (Table 4)	Post-Distillation	10.8	41.2	52	0.26:1

* Distilled at Porsgrunn.

As seen from the above results, Example 8 as replicated did not yield the 80-90% combined EPA+DHA ethyl ester concentration reported in the patent. It yielded only about a 52-55% concentration (true weight percent).

3) Conclusions: The foregoing results demonstrate that the procedure disclosed in Examples 2, 3, and 6-8 of Cornieri et al. for extracting polyunsaturated fatty acid esters from fish oils is operable to prepare concentrates containing up to about 55 weight percent EPA+DHA, but not the "at least 80% by weight" concentration that we are claiming in our application. The results also demonstrate that the most concentrated EPA+DHA mixtures obtained by the Cornieri et al. procedure have EPA:DHA weight ratios in the range of about 0.25:1 to 0.33:1, and not within the range of "1:2 to 2:1" that we are claiming in our application.

4) I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed by me to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of any patent that might issue on the above-identified application.

Porsgrunn, Norway

Date: _____

Harald Breivik

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3) Conclusions: The foregoing results demonstrate that the procedure disclosed in Examples 2, 3, and 4-6 of Cornieri et al. for extracting polyunsaturated fatty acid esters from fish oils is operable to prepare concentrates containing up to about 55 weight percent EPA+DHA, but not the "at least 80% by weight" that we are claiming in our application. The results also demonstrate that the most concentrated EPA+DHA mixtures obtained by the Cornieri et al. procedure have EPA:DHA weight ratios in the range of about 0.25:1 to 0.33:1, and not within the range of "1:2 to 2:1" that we are claiming in our application.

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Foragrunn, Norway

Date:

1994-08-12


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PS0107/6660/0000

CURRICULUM VITAE

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EDUCATION:

1976: M. Sc., University of Oslo, Department of
Organic Chemistry

1980: Ph. D., University of Oslo, Department of Organic
Chemistry. Exam in special curriculum, Medicinal Chemistry.

1985: First section of the Norwegian law degree, University of
Oslo.

SPECIALIST TRAINING:

American Oil Chemist's Society:

May 1986, Kona, Hawaii: Short Course on Marine Lipids and
EPA.

September 1987, King's Island Ohio: Short Course on
Oleochemicals.

The Center for Professional Advancement:

October 1986, Amsterdam: Course on Good Laboratory Practices

May 1987, Amsterdam: Course on Pharmaceutical Quality
Assurance and Control.

EMPLOYMENT:

- 1977-80: Research Associate, University of Oslo, Department of Chemistry.
- 1980-: Senior Scientist, Norsk Hydro a.s, Research Centre Porsgrunn, Porsgrunn, Norway. Currently head of Omega-3 fatty acids group.
- 1993-: Development Manager, Pronova Biocare a.s, Sandefjord, Norway.

PUBLICATIONS RELATED TO FATTY OILS AND DERIVATIVES:

H. Breivik, Some view-points regarding production and quality control of omega-3 concentrates, 16th Scandinavian Symposium on Lipids, June 1991, *Proceedings Lipidforum*, 1991 p. 301-305.

H. Breivik and K.H. Dahl, Production and Quality Control of n-3 Fatty Acids, In: J.C. Frölich and C. von Schacky (eds.) *Klinische Pharmakologie. Clinical Pharmacology* Vol. 5, *Fish, Fish Oil and Human Health*, 1992 W. Zuckschwerdt Verlag GmbH, Munich.

H. Breivik, N-3 Concentrates. A Scandinavian view-point. Compendium for invited lecture at the American Oil Chemist's Society Short Course: *Modern Applications of Marine Oils*, Toronto, Canada, May 1992. 39 pp.

T. Tande, H. Breivik and T. Aasoldsen, Validation of a Method for Gas Chromatographic Analysis of Eicosapentaenoic Acid and Docosahexaenoic Acid as Active Ingredients in Medicinal Products, *J. Am. Oil Chem. Soc.* 69:1124-30 (1992).

H. Breivik, S.E. Aakre, T. Kleivane and H.M. Næss, Stability of Omega-3 Concentrates. Results from Stability Studies. 17th Nordic Lipid Symposium, June 1993. *Proceedings Lipidforum* 1993 p. 184-185.

Holder of 3 patents and several patent applications in the lipid area.

PROFESSIONAL ASSOCIATIONS:

Member of: American Oil Chemist's Society, Scandinavian Forum for Lipid Research and Technology, Norwegian Chemical Society, Society of Chemical Industry, UK.

MISCELLANEOUS:

Appointed by the Norwegian Medicines Control Authority as Norwegian specialist to Group of Experts No. 13H, Fatty Oils and Derivatives, the European Pharmacopoeia Commission.

Active participation in, and organization of, Islandic/Norwegian workshops on analysis of marine lipids: Porsgrunn 1988, Reykjavik 1990, Sandefjord 1992.

Table 1

Porsgrunn 27/6 - 19/7 1994

Batch nr.	Step	Approximate Pressure (mbar)	Temp. (°C)	Flow (ml/h)	Res. (W%)	Dist. (W%)	EPA (A%)	DHA (A%)	DPA (A%)	EPA+DHA (A%)	EPA : DHA (A%)
raw material	0						14.1	10.4	2.6	24.5	1.36 : 1
851-4	1	$2-4 \times 10^{-2}$	108	500	69.3	30.7	20	17.4	3.4	37.5	1.15 : 1
851-11	2	1×10^{-3}	100	200	25.1	74.9	19.4	40	8.5	59.4	0.49 : 1
851-26	3	1×10^{-3}	95	150	74.4	25.6	13.1	49.4	10.9	62.5	0.27 : 1

Batch nr.	Step	EPA (W%)	DHA (W%)	EPA+DHA (W%)	EPA : DHA (W%)
851-11	2	11.3	26.1	37.4	0.43 : 1
851-26	3	6.5	27.3	33.8	0.24 : 1

Table 2

Porsgrunn 27/6 - 28/7 1994

Batch nr.	Step	Approximate Pressure (mbar)	Temp. (°C)	Flow (ml/h)	Res. (W%)	Dist. (W%)	EPA (A%)	DHA (A%)	DPA (A%)	EPA+DHA (A%)	EPA : DHA (A%)
raw material	0						14.1	10.4	2.6	24.5	1.36 : 1
851-27	1	$2-4 \times 10^{-2}$	108	500	69.6	30.4	19.9	17.1	3.8	37	1.16 : 1
851-29	2	1×10^{-3}	80	200	68.6	31.4	26	26.1	5.2	52.1	1.0 : 1
851-30	3	1×10^{-3}	80	200	84	16	27	30.5	6.13	57.5	0.89 : 1
851-31	4	1×10^{-3}	88	200	76.5	23.5	25.5	38.3	7.87	63.9	0.67 : 1
851-32	5	1×10^{-3}	88	200	83.4	16.6	22.4	44.4	9.2	66.8	0.5 : 1
851-33	6	1×10^{-3}	88	200	88	12	17.8	48.9	10.3	66.7	0.36 : 1
851-34	7	1×10^{-3}	88	200	89.1	10.9	14.5	51	11.2	65.5	0.28 : 1
851-39 I	8	urea fract.					16.8	62.9	12.2	79.7	0.27 : 1
851-39 II	9	4×10^{-3}	89	500	96.2	3.8	15.5	64.5	12.4	80	0.24 : 1

Batch nr.	Step	EPA (W%)	DHA (W%)	EPA+DHA (W%)	EPA : DHA (W%)
851-31	4	18.5	30.2	48.7	0.61 : 1
851-32	5	14.9	32.6	47.5	0.46 : 1
851-33	6	11.6	33.9	45.5	0.34 : 1
851-34	7	8.4	34	42.4	0.25 : 1
851-39 I	8	10.8	43	53.8	0.25 : 1
851-39 II	9	9.7	43	52.7	0.23 : 1

Table 3

Porsgrunn 27/6 - 2/8 1994

Batch nr.	Step	Approximate Pressure (mbar)	Temp. (°C)	Flow (ml/h)	Res. (W%)	Dist. (W%)	EPA (A%)	DHA (A%)	DPA (A%)	EPA+DHA (A%)	EPA : DHA (A%)
raw material	0						14.1	10.4	2.6	24.5	1.36 : 1
851-27	1	$2-4 \times 10^{-2}$	108	500	69.6	30.4	19.9	17.1	3.8	37	1.16 : 1
851-35	2	1×10^{-3}	88	200	54.6	45.4	26.4	31.4	6.3	57.8	0.84 : 1
851-42	3	4×10^{-3}	88	200	81.6	18.4	24.4	37.2	7.8	61.7	0.66 : 1
851-43	4	4×10^{-3}	88	200	81.1	18.9	21.7	44	9.3	65.6	0.49 : 1
851-44	5	4×10^{-3}	88	200	86.5	13.5	17.8	48.9	10.5	66.7	0.36 : 1
851-46 I	6	urea fract.					18.9	52.5	11.5	71.5	0.36 : 1
851-46 II	7	$2-4 \times 10^{-3}$	89	400	95.4	4.6	18.7	59.9	12.3	78.6	0.31 : 1

Batch nr.	Step	EPA (W%)	DHA (W%)	EPA+DHA (W%)	EPA : DHA (W%)
851-42	3	18.6	29.4	48	0.63 : 1
851-43	4	14.6	32.2	46.8	0.45 : 1
851-44	5	11	33.2	44.2	0.33 : 1
851-46 I	6	13.7	41.4	55.1	0.33 : 1
851-46 II	7	12.2	41.9	54.1	0.29 : 1

Table 4

Sandefjord 19/7 - 28/7 1994

Porsgrunn 28/7 - 29/7 1994 (Urea fractionation and Distillation: 851-41 I)

Batch nr.	Step	Approximate Pressure (mbat)	Temp. (°C)	Flow (ml/h)	Res. (W%)	Dist. (W%)	EPA (A%)	DHA (A%)	DPA (A%)	EPA+DHA (A%)	EPA : DHA (A%)
raw material	0										
Res.1	1	1×10^{-1}	108	500			20	17.2		37.2	1.16 : 1
Res.2	2	4×10^{-2}	78	500	89	11	21.1	19.2		40.3	1.10 : 1
Res.3	3	5×10^{-2}	88	500	79	21	24.7	24		48.7	1.03 : 1
Res.4	4	5×10^{-2}	88	500	85	15	23.9	26.7		50.6	0.90 : 1
Res.5	5	5×10^{-2}	88	500	86	14	24	29.9		53.9	0.80 : 1
Res.6	6	5×10^{-2}	88	500	89	11	23.1	32.3		55.4	0.72 : 1
Res.7	7	5×10^{-2}	88	500	90	10	22.7	36.9		59.6	0.62 : 1
Res.8	8	5×10^{-2}	88	500	94	6	21.2	35.7		56.9	0.59 : 1
Res.9	9	5×10^{-2}	88	500	92	8	18.9	39.9		58.8	0.47 : 1
Res.10	10	5×10^{-2}	88	500	95	5	18.1	38.9		57	0.47 : 1
Res.11	11	5×10^{-2}	88	500	93	7	17.4	43.7		61.1	0.40 : 1
Res.12	12	7×10^{-2}	88	500	99	1	18.9	44.6		63.5	0.42 : 1
Res.13	13	8×10^{-2}	88	500	94	6	15.7	45.3		61	0.35 : 1
Res.13	GLC analysis in Porsgrunn										
851-41 I	14	urea fract.					19	59.1	11.9	78	0.32 : 1
851-41 II	15	4×10^{-2}	89	500	94.6	5.4	17.2	61.6	12.3	78.8	0.30 : 1

Batch nr.	EPA (W%)	DHA (W%)	EPA+DHA (W%)	EPA : DHA (W%)
Res.13	10	33.4	43.4	0.30 : 1
851-41 I	12.2	40.9	53.1	0.30 : 1
851-41 II	10.8	41.2	52	0.26 : 1

Table 5

Sandefjord 27/6 - 11/7 1994

Batch nr.	Step	Feed (batch nr.)	Temp. (°C)	Flow (ml/h)	Res. (W%)	Dist. (W%)	EPA (A%)	DHA (A%)	EPA+DHA (A%)	EPA : DHA (A%)
raw material	0						14.1	10.4	24.5	1.36 : 1
Res:1	1	raw material	108	500	66	34	18	15.7	33.7	1.15 : 1
Res:2.1	2	Res:1	78	500	79	21	21.2	18.8	40	1.13 : 1
Res:2.2	2	Res:1	78	200	57	43	23.1	27.6	50.7	0.84 : 1
Res:2.3	2	Res:1	78	100	55	45	22.9	26.7	49.6	0.86 : 1
Res:2.4	2	Res:1	90	200	50	50	21.8	28	49.8	0.78 : 1
Res:2.5	2	Res:1	110	200	11	89	15.8	31.6	47.4	0.50 : 1
Res:2.6	2	Res:1	100	200	30	70	17.2	31.5	48.7	0.55 : 1
Res:3.1	3	Res:2.x	90	200	77	23	20.9	34.5	55.4	0.61 : 1
Res:3.2	3	Res:2.x	110	200	70	30	13.2	31.8	45	0.42 : 1
Res:4	4	Res:3.1	100	200	78	22	16.4	37.4	53.8	0.44 : 1

Res:2.x is a mixture of Res:2.2, Res:2.3, Res:2.4, and Res:2.6. (20.9% EPA, 29.0% DHA)

The pressure for the Step 1 distillation was approximately $1-2 \times 10^{-2}$ mbar.
For each of the subsequent distillations a pressure of approximately $4-6 \times 10^{-3}$ mbar was used.